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FRONTAL DECORTICATION AND ADAPTIVE CHANGES IN STRIATAL CHOLINERGIC NEURONS: NEUROPHARMACOLOGICAL AND BEHAVIORAL IMPLICATIONS

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19. ABSTRACT (Continue on reverse if necessary and identify by block number)
In neuropharmacological studies it was found that interruption of the corticostriatal pathway by undercutting the frontal cortex resulted after 2 weeks in marked reductions of basal ACh release in vivo determined by brain microdialysis and inhibition of the striatal sodium-dependent high-affinity uptake of choline (SDHACU). The lesion, too, completely prevented the rise in striatal ACh content induced by oxotremorine (OTMN) and R-apomorphine (APO). Acute i.p. injections of 100 mg/kg of either oxiracetam (OXI) or choline (Ch) resulted in time-dependent recovery of ACh output from the striata of decorticated (DC) rats to control levels. OXI also normalized the ex vivo striatal SDHACU activity of DC rats 2h after acute administration without exerting any effect in sham-operated rats. OXI or Ch administered before OTMN or APO reinstated the ACh-increasing effect of these agonists. In molecular mechanisms studies it was found that cortical deafferentation: 1), did not affect membrane fluidity and lipid phase transition of striatal synaptosomes and 2), produced a net reduction of [3H]-Hemicholinium binding sites as ascertained by saturation and autoradiographic studies. In behavioral studies a general pattern of disinhibition was obser-

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List of abbreviations: acetylcholine (ACh); choline (Ch); apomorphine (APO); oxotremorine (OTMN); OXI, oxiracetam; SDHACU, sodium-dependent high affinity choline uptake; PC, phosphatidylcholine; DC, decorticated; [^{3}H]HCh-3, [^{3}H]hemicolinium; APO, R-apomorphine.

STATEMENT OF WORK

The objective of this research project is to characterize the mechanisms whereby striatal cholinergic neurons in decorticated rats become refractory to agonists which depress cholinergic activity through receptor mediated responses. Three main lines of research are being pursued.

Neuropharmacological synaptic mechanisms: various drugs and lesions designed to interfere with transmission in known central aminergic pathways are being employed in an attempt to facilitate the neuropharmacological cholinergic action in striatum of decorticated rats.

Molecular mechanisms: molecular alterations in the striatal cholinergic neurons are being studied in terms of: 1), regulation of acetylcholine release in vivo and in vitro; 2), interaction of acetylcholine or cholinomimetics with their receptors; 3), signal transduction at the postsynaptic cell; 4), methylation in biological signal transmission in the membrane; 5), membrane fluidity.

Restoration studies: experiments aimed at restoring any defective activities related to cholinergic function are being pe rormed: i.e. addition of choline, mimetics of excitatory putative neurotransmitters, nootropic drugs, selective lesions.

Behavioral effects of frontal decortication: frontal decortication has important implications in behavioral responses to drugs. We have some preliminary data which show that the cataleptic behavior induced by narcotics, as well as pain mechanisms,

sleep-waking cycle, learning and memory processes and body temperature are altered by decortication. Narcotic catalepsy and learning and memory processes are the behaviors being studied first. Restoration studies: experiments to restore altered behaviors are being performed as described above.

It is envisioned that the information gleaned will enable us: 1), to pinpoint the functional organization of neuronal systems operating within the striatum; 2), to restore the activity of the cholinergic neurons intrinsic to the striatum in decorticated rats; 3), to provide insights into the cortical mechanisms regulating subcortical behavioral function. Furthermore, it should provide a means for understanding and potentiating normal mechanisms regulating CNS activity.

1. Interim Technical Report

i. Neuropharmacological synaptic mechanisms

Restoration studies: Interruption of the corticostriatal pathway by undercutting the cortex resulted after two weeks in a 40% reduction of acetylcholine (ACh) release in vivo (from 8.4 ± 0.6 to 5.3 ± 0.9 pmoles/10 min) (Fig. 1), a degree consistent with both the impairment of the sodium-dependent high-affinity uptake of choline (SDHACU) and the rate of ACh synthesis (see previous Reports). The lesion rendered striatal cholinergic neurons refractory to the inhibitory pharmacological actions of OTMN and APO which increase ACh content directly through respective muscarine and dopamine receptor actions (see previous Reports). The defective biochemical and pharmacological activities of these neurons were not a reflection of physical damage to the striatal cells possibly induced by decortication since choline acetyltransferase, a specific marker for cholinergic neurons, was not altered by the lesion. In addition, the increase in striatal ACh elicited by a high dose of choline (Ch) was not blocked in decorticated (DC) rats. We further reported that the marked lowering in striatal ACh content induced by antagonists such as the typical and atypical neuroleptics and the antimuscarinic agent scopolamine was not influenced by the lesion. In the light of these findings, it was proposed that the lesion altered the sensitivity state of the cholinergic neurons upon which the inhibitory effects of the drugs depend.

However, there is some evidence that this group of neurons, even when its activity is depressed, remains potentially functional and capable of a response to certain stimuli. The striatum of DC rats could thus constitute a useful model for studying means to restore deficits in cholinergic neurotransmission. We then investigated whether Ch, a precursor of ACh and oxiracetam (OXI), a psychic energizer in the 2-pyrrolidinone chemical class, could promote the recovery of the basic biochemical cholinergic processes and the cholinergic neuropharmacological activity of APO and OTMN in striata of DC rats (please see section B.4 A1-A2 and B.1 of Research Proposal, 17 Dec. 1985).

i.a. ***Restoration of ACh release in vivo by OXI or Ch in DC rats:*** The extracellular ACh content in vivo was measured in striata of 14-day DC and sham-operated rats, using the microdialysis technique. The results are shown in **Fig.1.** The ACh levels in the 10 min perfusate samples are given as values uncorrected for the recovery which was 49.2% for a probe 7.5 mm long.

The ACh output was constant over at least 240 min in the sham-operated and DC rats. The average ACh content in the perfusate of sham-operated rats was 8.1 ± 0.2 pmoles/10 min and this was significantly lowered, to 5.3 ± 0.9 pmoles/10 min ($p < 0.01$), in DC rats given saline.

An intraperitoneal dose of OXI (100 mg/kg) did not significantly

alter the ACh output from striata of sham-operated rats at any time over the entire collection period; the average ACh content for this group (sham+OXI) was not significantly different (8.4 ± 0.6 pmoles/10 min) from the sham-operated group of rats given saline, so only the ACh release curve of sham + OXI is presented in **Fig 2.**

In DC rats, the nootropic drug induced a time-dependent recovery of ACh output from the striata. Starting 30 min after drug injection, ACh release was already significantly ($p<0.01$) higher in this group of lesioned rats than in the group of lesioned animals receiving saline, and the rate of ACh release continued to rise gradually, with complete recovery about 80 min post-OXI injection. The full effect lasted at least 100 min longer.

Pretreatment with Ch (100 mg/kg i.p.), the ACh precursor, did not influence the ACh output from striata of sham-operated rats but completely overcame the reduction in extracellular ACh observed in DC rats **(Fig. 3)**. A substantial recovery effect started 10 min after Ch administration when the ACh content of treated lesioned rats was 36% higher than in untreated lesioned ones. ACh release appeared to plateau about 30 min after the start of treatment, when control values were reached. Ch's effect lasted at least 80 min more.

i.b. ***Restoration of SDHACU activity by OXI in DC animals:***

The SDHACU by the P_2 fraction of striatal homogenates was reduced by about 35%, 14 days after frontal decortication compared to the

sham operated animals (from 0.8 ± 0.06 to 0.5 ± 0.03 nmoles Ch taken up /min /g protein **(Table 1).** OXI normalized the striatal SDHACU activity of DC rats 120 min after a dose of 100 mg/kg, but had no effect on SDHACU activity of sham-operated rats.

i.c. ***Restoration of the ACh increasing effect of OTMN and APO In DC rats by OXI or Ch:*** OTMN, a typical muscarinic agonist, at the dose of 0.5 mg/kg i.p., 20 min, and APO, a D_1-D_2 dopaminergic agonist, at the dose of 1.0 mg/kg, i.p., 30 min, increased striatal ACh content in sham-operated rats by about 30%. Decortication by itself did not affect striatal ACh content but it completely prevented the increase in cholinergic effect of both OTMN and APO **(Fig. 4).**

OXI, 100 mg/kg, i.p., 120 min, did not by itself affect striatal ACh content in either sham-operated or DC rats, but when it was administered prior to OTMN and APO (100 and 90 min, respectively) it restored the ACh increasing effect of these drugs.

In a dose-response study, it was found that at the higher dose of 300 mg/kg, OXI had a similar restorative effect on the OTMN and APO-induced ACh increase, but not at the lower doses of 30 and 60 mg/kg. By itself, OXI affected neither striatal ACh nor the striatal Ch content in either sham-operated or DC rats 120 min after any of the doses tested.

Ch, 100 mg/kg, given 10 min before OTMN or APO reinstated the ACh-increasing effect of the two agonists in DC rats **(Fig. 5)**. At

the dose of 100 mg/kg, 10 min, Ch did not significantly affect striatal ACh in sham-operated or in DC animals (data not shown) but raised Ch content about 60 % in both groups **(Table 2).** The effect was transient since at 30 and 80 min it was no longer detectable.

The data taken together indicate that Ch most likely acts directly simply by being the precursor for ACh whereas OXI appears to act indirectly, possibly by increasing the availability of Ch for ACh synthesis. In accordance with this hypothesis is the finding (Trovarelli et al., 1986) that OXI improves the in vitro and in vivo synthesis of phospholipids impaired by aging, and in particular, the phosphatidylcholine (PC) pool proposed as a "reservoir" to generate choline for the synthesis of ACh (Blusztajn et al., 1986; Lakher et al., 1986). Interestingly, it was reported that ACh can be synthesized from Ch derived from the breakdown of endogenous PC formed *de novo* by the stepwise methylation of phosphatidylethanolamine (Bremer et al., 1960). In accordance with this is the finding that in striatum of DC rats, the transmethylation pathway is enhanced (Tacconi et al.,1988), possibly to sustain the striatal cholinergic activity depressed by the lesion. However, whether the effect of OXI occurs in the transmethylation pathway or in others known to exist in the brain (Blusztajn et al., 1986; Lakher et al., 1986), e.g. CDP-choline and the base exchange pathways, needs to be clarified.

In summary, our findings indicate that the loss of ACh release in vivo and the SDHACU activity in DC rats can be reversed with OXI and

Ch. This repletion is most likely associated with enhanced ACh formation. As a consequence, the cholinergic neuropharmacological effects of APO and OTMN are also reinstated in DC rats.

i.d. ***Restoration of the ACh increasing effect of OTMN and APO In DC rats by the nootropic drugs piracetam and aniracetam:*** Other nootropic agents, piracetam and aniracetam, belonging to the same chemical class as OXI, were tried in decorticated rats. Piracetam, 100 mg/kg, i.p., 120 min, did not by itself affect striatal ACh content in either sham-operated or DC rats, but when it was administered prior to OTMN and APO (100 and 90 min, respectively) it restored the ACh increasing effect of these drugs **(Table 3-4)**. Similarly, aniracetam 100 mg/kg, orally, 120 min, in the same conditions as piracetam, allowed OTMN and APO to express their full inhibitory effects on the cholinergic neurons without having an effect by itself **(Table 5-6).** The restoring effect of aniracetam was evident also at the lower dose of 30 mg/kg (data not shown).

ii. Molecular mechanisms in decorticated rats

ii.a. ***Effect of cortical deafferentation on striatal synaptosome fluidity.*** Surgical undercutting of cortical afferents and subsequent interruption of excitatory input increased phospholipid methylation in striatal synaptosomes as early as 3 days after lesion and the effect lasted at least 60 days (Tacconi et

al., 1988; see Interim Technical Report Nov. 7, 1988). As a consequence of phospholipid methylation increase, more monomethylphospholipids are generated which has been reported to lead to decreased membrane viscosity (Masturzo et al. 1985). Thus, it was tested whether membrane viscosity was altered in decorticated rats.

Striata from 14 days decorticated and sham-operated rats were homogenized in 0.32 M sucrose and then synaptosomes were prepared by gradient centrifugation according to the method of Dodd at al., (1981). Aliquots of synasptosomes were used to measured fluorescence polarisation, as an index of membrane fluidity, with a microviscosimeter Elscint MV 1, (Haifa, Israel) using 1-6 diphenylhexatreiene as fluorescent probe.

In a first experiment, membrane microviscosity was measured at constant temperature (37°), **Table 7.** No differences were found between decorticated and sham-operated synaptosomes. We then measured microviscosity's changes with temperature, from 20° to 42° and Arrhenius plots were drawn **(Fig. 6).** Decortication did not influence the slope of the temperature curve as compared to sham-operated rats.

We conclude that membrane fluidity and lipid phase transition of striatal synaptosomes are not affected by interruption of cortical afferents to the striatum.

It would be of interest to further determine whether drug inducing effects on membrane fluidity would be afffectd by deafferentation.

It was previosly shown that the muscarinic agonist, OXO, produced a linear dose-dependent increase in membrane fluidity of intact and viable human lynphocytes in vitro; particularly since the action of this drug on striatal ACh content is prevented by decortication.

ii.b.***Effect of cortical deafferentation on [^{3}H]hemicholinium binding and its brain distribution in DC rats.*** Hemicholinium is a potent and reversible inhibitor of the sodium-dependent high affinity choline uptake (SDHACU). The availability of [^{3}H]hemicholinium ([^{3}H]HCh-3) of high specific activity has allowed to measure the binding of this ligand to the carrier sites for the SDHACU.

It was thus of interest to examine, by saturation experiments, whether the binding characteristics of [^{3}H]HCh-3 to SDHACU sites of rat striatal and hippocampal membranes are affected by frontal deafferentation. In addition, in a neuroanatomical context, we have studied the distribution of [^{3}H]HCh-3 sites.

Saturation experiments. Striata and hippocampi from two weeks lesioned animals were dissected bilaterally and crude membranes obtained by sonication were centrifuged at 20,000 g for 15 min at 4°C. The resulting pellets were washed twice prior to resuspension in glycylglycine buffer pH 7.8 to yield a final concentration between 200 and 800 µg/ml, used in the binding assay. The binding of [^{3}H] HCh-3 [NEN, USA; 5457.5 GBq/mmol (147.5 Ci/mmol)] was performed as described by Sandberg and Coyle (1985) with minor modifications;

non-specific binding was defined as the binding in the presence of 1 μM unlabelled HCh-3.

A saturation curve of [^{3}H]Ch-3 binding to striatal membranes obtained from sham-operated and deafferentated rats is shown in **Fig 7**. In the striatum of the sham-operated group, the number of binding sites (B_{max}) and the affinity (K_D) were 170 ± 20 fmol/mg protein and 7.2 ±1.7 nM, respectively. The lesion produced a clear reduction of about 30% in the B_{max} whereas no change in the K_D was observed **(Table 8)**. After frontal deafferentation neither binding parameter was modified in the hippocampal region, being the Bmax = 21 fmol/mg protein and the K_D = 6.1 nM.

Autoradiographyic experiments : The whole brain was removed and rapidly frozen. Coronal and sagittal sections were cut at -20°C in a cryostat and thaw mounted onto acid cleaned, gelatin subbed slides. The slides were incubated with 10 nM [^{3}H]HCh-3 for 10 min at room temperature in 50 mM glycylglycine buffer pH 7.8 containing 200 mM NaCl. Non specific binding was determined in adjacent sections processed in the same manner except that 10 μM unlabelled HCh-3 was added to incubation medium. After dessication, the slides were exposed to tritium-sensitive film "Hyperfilm" (Amersham, UK) for 4 weeks and developed using standard technique. Tritiated microscales (Amersham) were coexposed. Quantitative autoradiographic analysis was performed by RAS 1000 Image Analysis System.

Table 9 summarizes the data attained from the image analysis.

The bilateral deafferentation produced a significant decrease (30%) of the [^{3}H]HCh-3 binding sites in the anteromedial part of striatum, where the signal was higher. A similar reduction was found also in striata of unilaterally lesioned rats. No change was detected in the accumbens-olfactory tubercle or in the posterior part portion of striatum.

iii) Behavioral Effects of Frontal Decortication: Learning and memory processes

In order to identify (please see section B.4 C, research proposal, 17 Dec. 1985) the functional consequences of disconnecting basal ganglia from the cortex, the behavior of control and lesioned animals has been examined in different situations:

iii. a. ***Open field and reactivity to novelty***

iii. b. ***Lashley maze***

iii. c. ***Active avoidance learning***

iii.a.*Open field and reactivity to novelty:* Bilaterally-lesioned (n=9) and control (n=9) rats were firstly examined in an open field situation (5 min) and successively tested for reactivity to novelity (3 min) by introducing a plexiglass box (novel object) in the same apparatus. The following parameters were recorded: number of crossed squares (peripheral or central), number of rearings (against the wall or not), contact with object (sniffing and rearing against), boluses. For each parameter, the mean scores recorded in each group

together with the value of the statistical comparison are reported in **Table 10.** The results show that lesioned animals cross significantly more squares (central as well as peripheral) than controls. This increased explorative behavior is accompained by a significantly higher number of rearings against the walls. Finally lesioned animals also tend to develop more interactions with the novel object (marginally significant effect). The behavioral pattern expressed by lesioned animals in this situation can therefore be ascribed to a general disinhibition which increases subject environment interactions.

iii.b. ***Lashley maze*** : Two groups of rats (bilaterally operated and control) were then tested in the Lashley maze, a simple spatial task. The maze consists in three parallel and adjacent arms. The access from one arm to the other is warranted by a door allowing a transversal crossing of the apparatus, from the starting box until the goal box **(Fig. 8)**. An error is counted each time the rat retraces or goes beyond the door. The animals are allowed to make one run for three consecutive days. Two dependent variables are recorded: (1) the number of errors (2) the running time. Between group differences were estimated by a two factor (lesion x days) ANOVA. The results shows that bilaterally-lesioned animals solve better the task than sham-operated. Both the numbers of errors ($F_{1/22}$=6.2 $p<0.02$) and the running times ($F_{1/22}$ = 4.6 $p<0.04$) are significantly lower in operated animals than in controls. Since errors in this task do not

only reflect defects in orienting but can also indicate a high rate of emotionality (the rat retraces towards the starting box which has a status of nest), it can be assumed that operated animals, which make few errors and run the maze quickly, are less stressed than controls when placed in this situation. Their behavior is consistent with the behavioral disinhibition observed in the open field.

iii. c. ***Active avoidance learning.*** Associative abilities were then investigated by submitting three groups (n=12) of subjects (sham-bilaterally and unilaterally-operated rats) to active avoidance problem. Avoidance training was carried out in automatic shuttle-boxes (Ugo Basile). A sound signal (30 dB) was used as conditional stimulus (CS) and an alternating current (60V) as unconditional stimulus (US). The CS (3 sec) preceded the onset of the US (3 sec) which in turn is followed by a pause (24 sec). The rats were subjected to ten daily 20-trial avoidance sessions. The animals were then left without training for 5 days. On the 16th day, a reminding session was carried out. After another 5 days training interruption, the animals were subjected to a second reminding session (22nd day). Finally, the sham-operated group was divided into two subgroups: animals from one subgroup (n=6) were unilaterally operated while animals from the second subgroup (n=6) served as controls. After a recovery period of 12 days, these animals were subjected to five daily 20-trial sessions. The results show that sham-operated rats reach an average of 90% avoidance

responses at the end of training. Unilaterally-operated rats perform lower than sham-operated since they reach an average of 50% avoidance responses only. Finally, bilaterally-operated rats were unable to develop the avoidance reflex. Similar differences are also found in analyzing reaction times to the CS. Sham-operated rats cross from one compartment to the other soon after the onset of the CS while latencies in unilaterally operated animals are higher **(Fig. 9)**.

In conclusion, cutting unilaterally the connections between cortex and basal ganglia leads to marked deficits in active avoidance conditioning while the bilateral operation completely abolishes this reflex. Interestingly, the impairment observed in bilaterally operated rats could be, at least partly, interpreted in the light of the results obtained in the other situations. The fact that no crossing in this group corresponds to an avoidance response, in spite of the increased activity displayed by those animals in the open field, suggests that the performance deficits cannot be explained in terms frustration effect or defects in arousal. Moreover, the general pattern of behavioural disinhibition, particulary evidenced by a lack of stressful reactions in the presence of the novel object and the low running times in the Lashley maze, may have lowered the emotionality level of bilaterally-operated animals, which could be less reactive to the nociceptive unconditioned stimulus and consequently impaired in active avoidance conditioning. This hypothesis must be examined by controlling the reactivity

thresholds to electric shocks in all groups. Nevertheless, specific memory deficits could also be responsible for the performance impairments observed in these experiments. In order to investigate this point, we have in project to compare lesioned and sham-operated rats in more complex learning and memory tasks. In particular, testing spatial learning and memory in the radial maze could be relevant for our purpose since the solving of this task requires both the memorization of visited places and the organization of foraging strategies. Consequently, performance deficits in this task would help us to characterize the effect of the lesion at the mnemonic level and more generally on cognitive processes.

2. Status of the Research

The work proposed for the period 29 Sept. 1988-29 Sept. 1989(please refer to pp 18 of Interim Technical Report November 1988) and overall aims and scope of this project have been essentially met.

3. Interactions (Coupling Activities)

After the sudden death of Prof. Luigi Valzelli contact was made with Dr. Martine Amassari-Teule of the istituto di Psicobiologia e Psicofarmacologia, National Research Council, Via Reno 100198 Roma who is now consultant advisor.

TABLE 1 - Oxiracetam restores SDHACU activity in striata of bilaterally decorticated rats.

	SDHACU (nmoles Ch/min/g P)	
	Sham	**DC**
Vehicle	0.8±0.06	0.5±0.03[a]
OXI	0.7±0.06	0.9±0.06

The experiments were done 14 days after bilateral frontal decortication. OXI , 100 mg/kg, was administered i.p. 120 min before Ch uptake was measured, as described in Materials and Methods. The data are means ± S.E.M. of 8 animals. a, $p<0.01$ vs sham group. $F_{1/25}=22.8$. ANOVA (2x2) and Tukey's test for unconfounded means.

TABLE 2 - Time-course of the effect of choline on striatal Ch content in sham-operated and in DC rats.

Time after choline (min)	Ch content (nmoles/g tissue)	
	Sham	DC
0	25.2 ± 1.4	23.7 ± 3.0
10	41.2 ± 4.0[a]	35.7 ± 4.0[a]
30	29.5 ± 1.8	26.6 ± 3.4
80	27.5 ± 1.8	28.2 ± 1.3

The data are means ± S.E.M. of 6 animals. Ch was administered i.p. at the dose of 100 mg/kg. [a]$p<0.01$ vs respective control group, Dunnett's test.

TABLE 3 - Restoration of the ACh increasing effect of oxotremorine by piracetam in DC rats.

	Striatal ACh content (nmoles/g tissue)				
	Sham	DC	OTMN	OTMN+DC.	F.int.
Vehicle	66.9±1.0	59.0±1.9	84.5±2.6**	62.0±2.1	14.1 p<0.01
Pirac.	67.2±1.5	64.2±2.0	84.7±2.4**	82.2±1.8**	n.s.

The experiment was done 14 days after unilateral frontal deafferentation. The animals were sacrificed 2 hrs after piracetam, 100 mg/kg i.p., and 20 min after OTMN 0.5mg/kg i.p. The drugs were dissolved in double distilled water. The data are means ± S.E.M. (n=12-14 rats). **p< 0.01 vs sham group; ANOVA (2x2) and Tukey 's test for unconfounded means.

TABLE 4 - Restoration of the ACh increasing effect of apomorphine by piracetam in DC rats.

	Striatal ACh content (nmoles/g tissue)				
	Sham	DC	APO	APO + DC.	F.int.
Vehicle	68.4±1.3	70.8±0.7	85.7±2.2**	69.7±1.4	12.9 p<0.01
Pirac.	70.4±1.9	71.9±1.8	87.9±2.2**	86.4±1.7**	n.s.

The experiment was done 14 days after unilateral frontal deafferentation. The animals were sacrificed 2 hrs after piracetam, 100 mg/kg i.p., and 30 min after APO 0.8 mg/kg i.p. The drugs were dissolved in bidistilled water. The data are means ± S.E.M. (n = 6-8 rats).
**p<0.01 vs sham group; ANOVA (2x2) and Tukey 's test for unconfounded means.

TABLE 5 - Restoration of the ACh increasing effect of OTMN by aniracetam in DC rats.

	Striatal ACh content (nmoles/g tissue)				
	Sham	DC	OTMN	OTMN+DC.	F.int.
Vehicle	67.4±1.1	64.1±0.9	87.4±2.1*	69.5±2.0	17.9 p<0.01
Anirac.	64.8±1.0	64.4±1.5	89.1±1.8*	92.1±4.9**	n.s.

The experiment was done 14 days after unilateral frontal deafferentation. The animals were sacrificed 2 hrs after aniracetam, 100 mg/kg p.o., and 20 min after OTMN 0.5 mg/kg i.p. Aniracetam was suspended in 0.5% CMC, OTMN was dissolved in doublebidistilled water. The data are means ± S.E.M. (n = 6-7 rats).** p< 0.01 vs sham group; ANOVA (2x2) and Tukey's test for unconfounded means.

TABLE 6 - Restoration of the ACh increasing effect of apomorphine by aniracetam in DC rats.

	Striatal ACh content (nmoles/g tissue)				
	Sham	DC	APO	APO+DC.	F.int.
Vehicle	67.0±1.0	71.3±0.6	86.5±2.5**	69.5±1.1	12.4 p<0.01
Anirac.	68.2±2.0	67.2±2.1	87.4±2.8**	94.0±4.4**	n.s.

The experiment was done 14 days after unilateral frontal deafferentation. The animals were sacrificed 2 hrs after aniracetam, 100 mg/kg p.o., and 30 min after APO 0.8 mg/kg i.p. Aniracetam was suspended in 0.5% CMC, OTMN was dissolved in bidistilled water. The data are means ± S.E.M. (n=6-7 rats). ** p< 0.01 vs sham group; ANOVA (2x2) and Tukey 's test for unconfounded means.

TABLE 7 - Effect of decortication on synaptosomal microviscosity measured at 37°C.

	Striatal Synaptosomal Microviscosity (poise)
Sham	**2.67 ± 0.05**
DC	**2.61 ± 0.02**

The data represent the means ± S.E.M. (n=6-8 experiments).

TABLE 8 - Effect of frontal deafferentation on the [^{3}H]HCh-3 binding to the SDHACU sites in striatum.

	Sham-operated	DC
B_{max} (fmol/mg P)	170 ± 20	120 ± 10*
K_D (nM)	7.2 ± 1.7	7.3 ± 1.1

The data are the means ± S.E.M. of 4-6 determinations *p <0.01, student's t test. The rats were killed 2 weeks after bilateral deafferentation. The parameters are derived from the "non-linear fitting" analysis of the saturation curves (1-24 nM).

TABLE 9 - Effect of decortication on the brain distribution of [^{3}H]HCh-3 binding sites determined by autoradiography.

	[^{3}H]HCh-3 binding sites (fmol/mg P)	
	Sham-operated	**DC**
STRIATUM		
Anteromedial portion	**207.6±20.2**	**141.8±4.5***
Posterior portion	**158.8±8.2**	**162.7±10.1**
NAS-TO	**187.8±8.7**	**170.3±23.9**

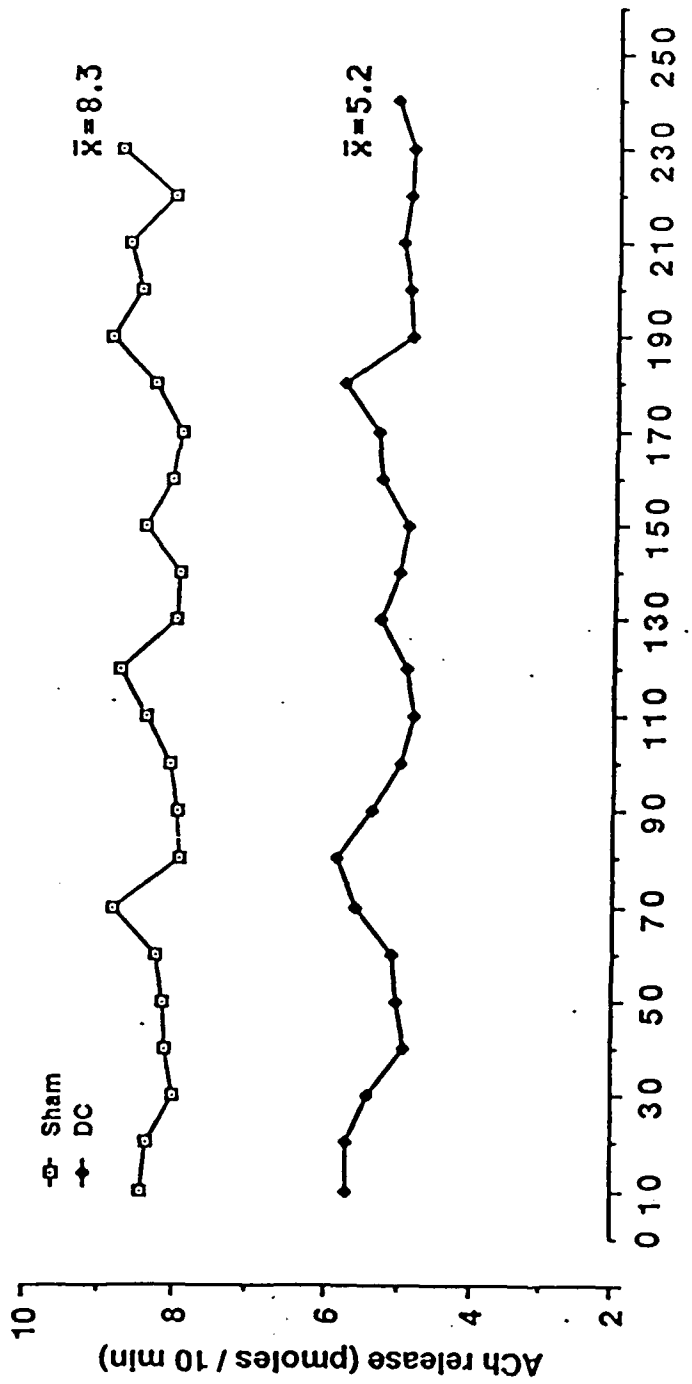
The data are the means ± S.E.M. of 4 rats *$p < 0.01$, student's t test. The rats were killed 2 weeks after bilateral deafferentation. A single concentration (10 nM) of [^{3}H]HCh-3 was used. NAS-TO, nucleus accumbens-tuberculum olfactorium.

TABLE 10- Exploratory behavior in DC and sham operated rats.

	SHAM	DC
Central crossing	0.8 ± 0.4	4.0 ± 1.0*
Peripheral crossing	8.7 ± 2.5	60.1 ± 4.6*
Rearing wall	4.1 ± 0.9	15.1 ± 1.3*
Rearing	0.8 ± 0.8	2.2 ± 0.7
Contact with object	0.2 ± 0.1	1.4 ± 0.4
Boluses	3.5 ± 1.0	5.0 ± 2.6

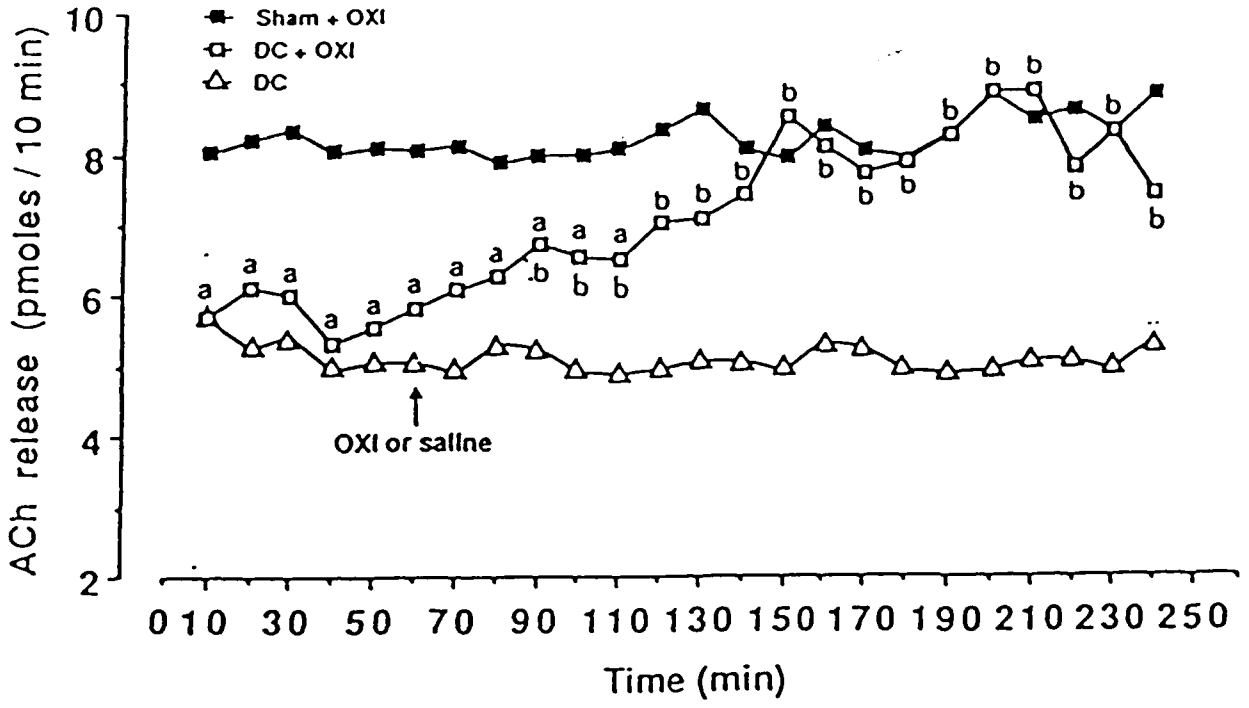
The data are the mean scores ± S.E.M. of 9 rats. Each animal was tested for a 5 min period Statistic: Mann-Whitney's test *$p \leq 0.05$ vs sham.

FIG. 1



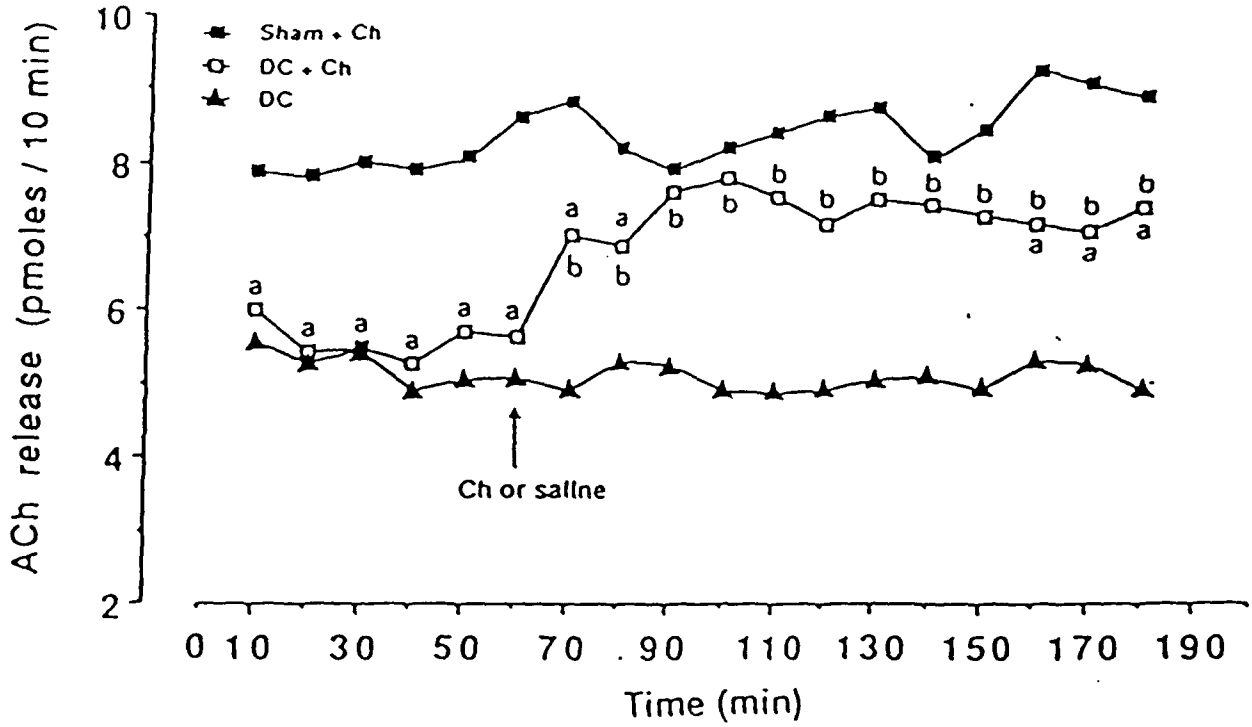
The experiments were done 14 days after sham operation or bilateral frontal decortication and 24 h after dialysis tube implantation. Perfusate was collected for 240 min. The data points (mean of 6 rats) represent the ACh content in each 10-min fraction and are expressed as pmoles/10 min. Variability between values did not exceed 5%. ACh release in the DC group was significantly ($p<0.01$) different from that of the sham group at all times as determined by Splitplot test and Tukey's test for unconfounded means.

FIG. 2



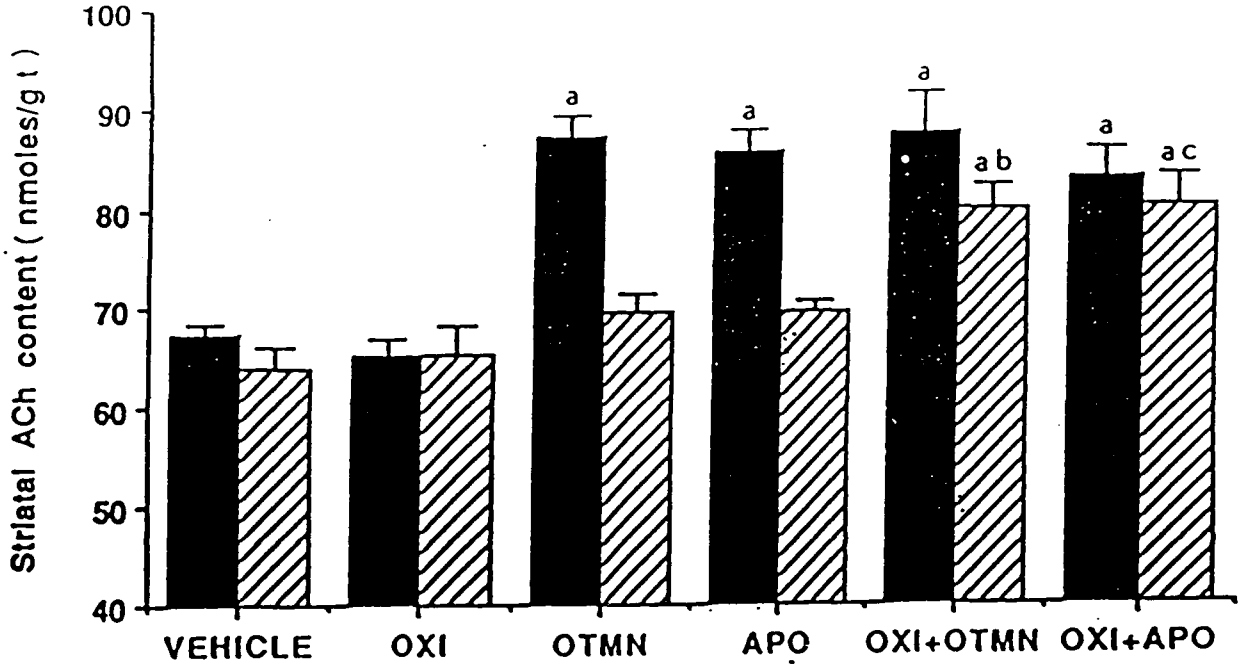
. OXI administration restores normal striatal ACh release in vivo in decorticated (DC) rats.The experiments were done 14 days after sham operation or bilateral frontal decortication and 24 h after dialysis tube implantation. Perfusate was collected for 60 min (6X10 min fractions) before injection of OXI 100 mg/kg i.p. or saline (shown by the arrow). The data points (mean of 6 rats) represent the ACh content in each 10-min fraction and are expressed as pmoles/10 min. Variability between values did not exceed 5%. ACh release in the sham + OXI group was not significantly different from that of sham + saline at any time (curve not shown). ACh release in the DC group was significantly ($p<0.01$) different from that of the sham + OXI group at all times as determined by Dunnett's test. Interactions: DC+OXI vs sham+OXI, $ap<0.01$; DC+OXI vs DC+saline, $bp<0.01$; Splitplot test and Tukey's test for unconfounded means.

FIG. 3



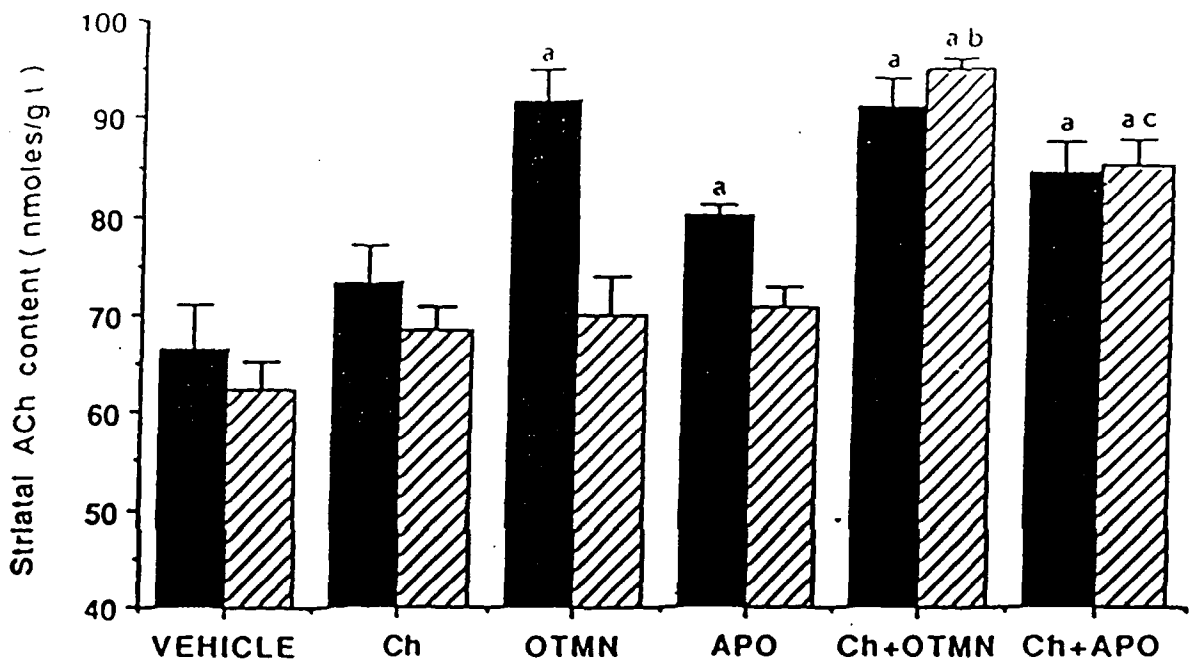
Ch administration restores striatal ACh release in vivo in decorticated (DC) rats. The experiments were done 14 days after sham operation or bilateral frontal decortication and 24 h after dialysis tube implantation. Perfusate was collected for 60 min (6X10 min fractions) before injection of Ch 100 mg/kg i.p. or saline (shown by arrow). The data points (mean of 6 rats) represent the ACh content in each 10-min fraction and are expressed as pmoles/10 min. Variability between values did not exceed 5%. ACh release in the sham + Ch group was not significantly different from that of sham + saline at any time (curve not shown). ACh release of DC group was significantly ($p<0.01$) different from the sham + Ch group at all times as determined by Dunnett's test. Interactions: DC+Ch vs sham+Ch, $^{a}p<0.01$; DC+Ch vs DC+saline, $^{b}p<0.01$, Splitplot test and Tukey' s test for unconfounded means.

FIG. 4



Blockade of the ACh-increasing effect of OTMN and APO in striata of decorticated (▨) rats and restoration by OXI. The experiments were done 14 days after unilateral (right side) frontal decortication. The animals were killed by microwave irradiation to the head 120 min after OXI,100 mg/kg i.p., 20 min after OTMN 0.5 mg/kg i.p. and 30 min after APO 1 mg/kg i.p. and the right striata were removed for determination of ACh content. The data are means ± S.E.M. (n = 6-12 rats). Data were analyzed statistical by two-way ANOVA followed by Tukey's test for unconfounded means; $^{a}p<0.01$ vs respective vehicle treated group. Interactions: OXI+OTMN vs OTMN, $F_{1/40} = 11.7$, $^{b}p<0.01$; OXI+APO vs APO, $F_{1/24} = 17.9$, $^{c}p<0.01$.

FIG. 5



Blockade of the ACh-increasing effect of OTMN and APO in decorticated (▨) rats and restoration by Ch. The experiments were done 14 days after unilateral frontal decortication. Ch, 100 mg/kg i.p., was administered 10 min before either OTMN 0.5 mg/kg i.p. or APO 1 mg/kg i.p. The animals were killed by microwave irradiation to the head 20 min after OTMN and 30 min after APO. The data are means ± S.E.M. (n=6-8 rats). Data were analysed statistically by two-way ANOVA followed by Tukey's test for unconfounded means; $^{a}p<0.01$ vs respective vehicle treated group. Interactions: Ch+OTMN vs OTMN, $F_{1/24}=15.1$, $^{b}p<0.01$; Ch+APO vs APO, $F_{1/16}=14.9$, $^{c}p<0.01$.

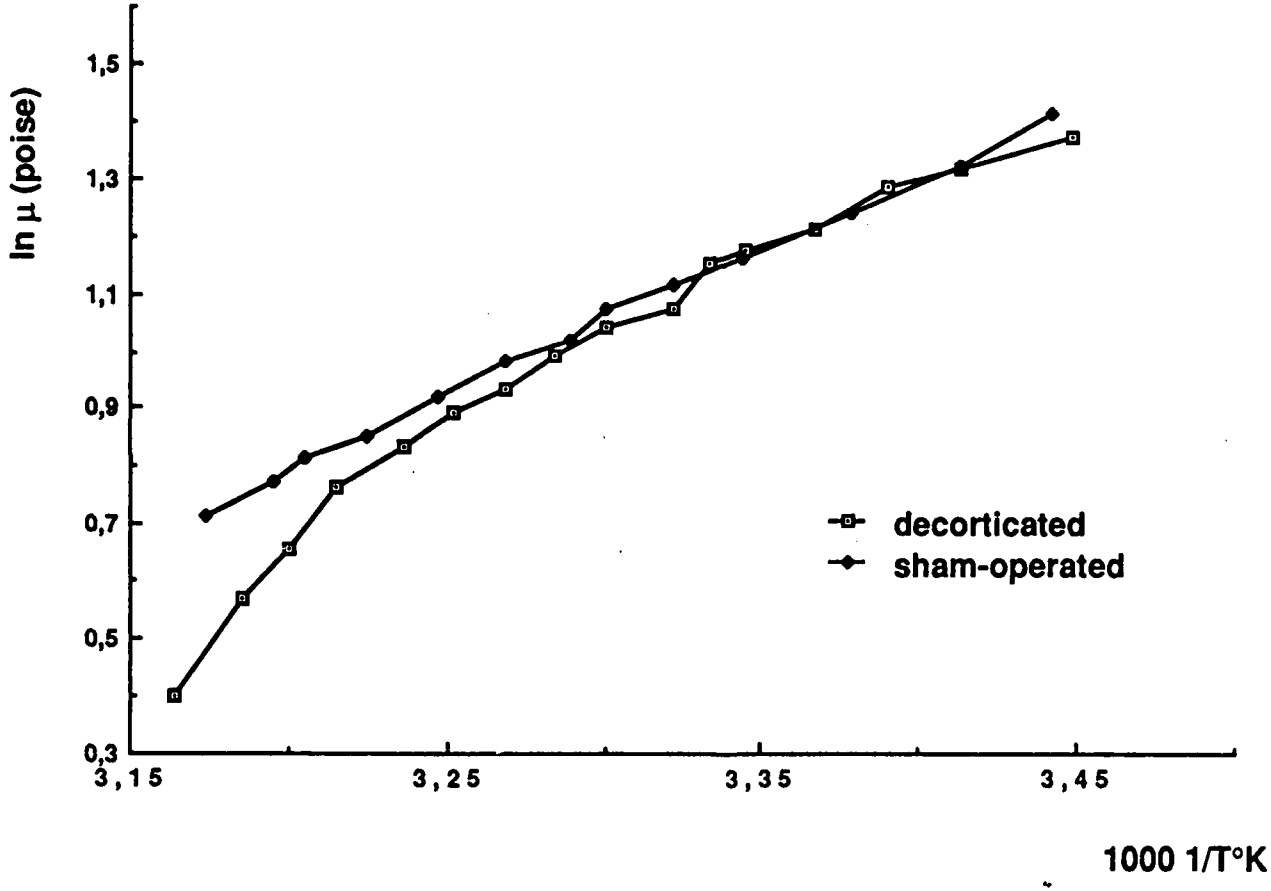


Fig. 6- Arrhenius plots of the microviscosity of striatal synaptosomal preparations from decorticated (-▫-) and sham-operated (-◆-) rats. Experiments were perfomed at 20-42°C. Each point represents the mean and of 6-7 rats.

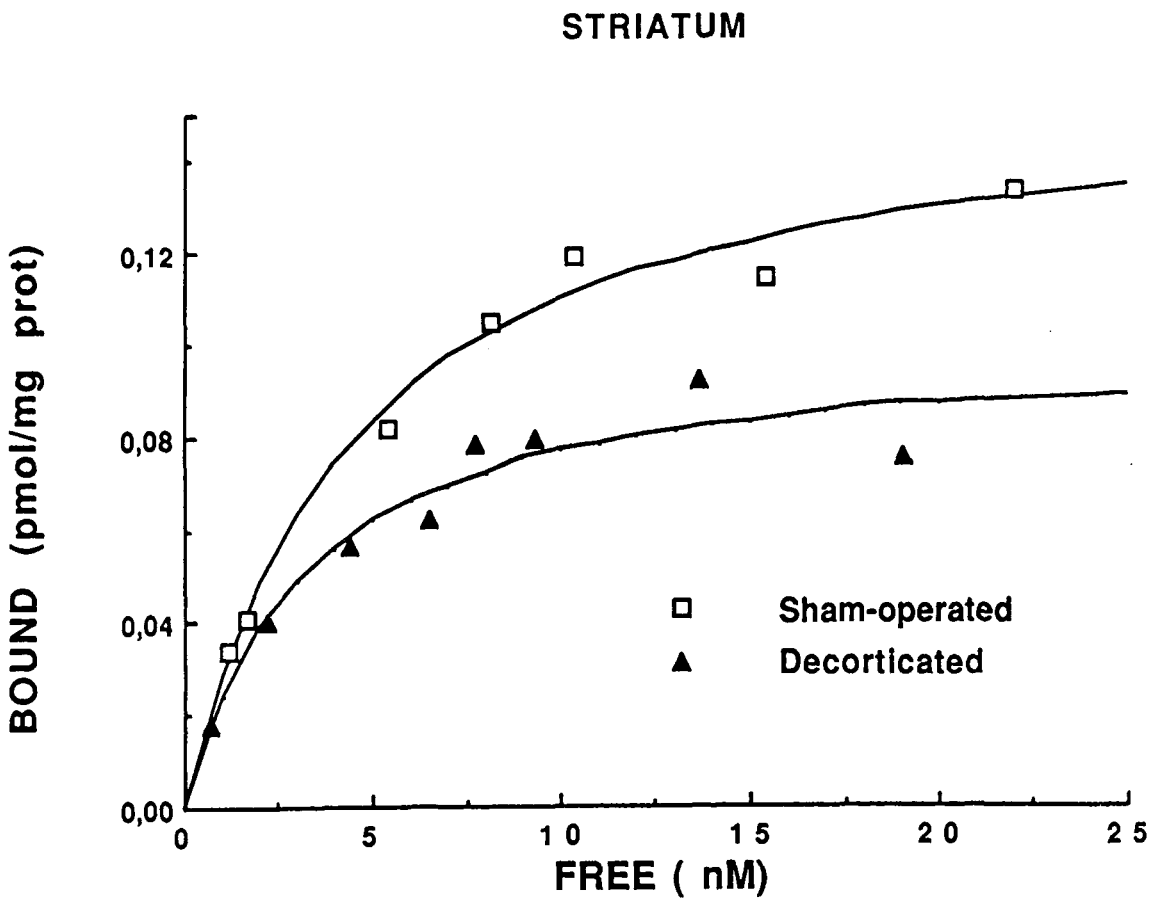


Fig. 7- Saturation curves of (1-24 nM) [^{3}H]HCh-3 binding to striatal membranes from sham-operated and decorticated rats

FIG. 8

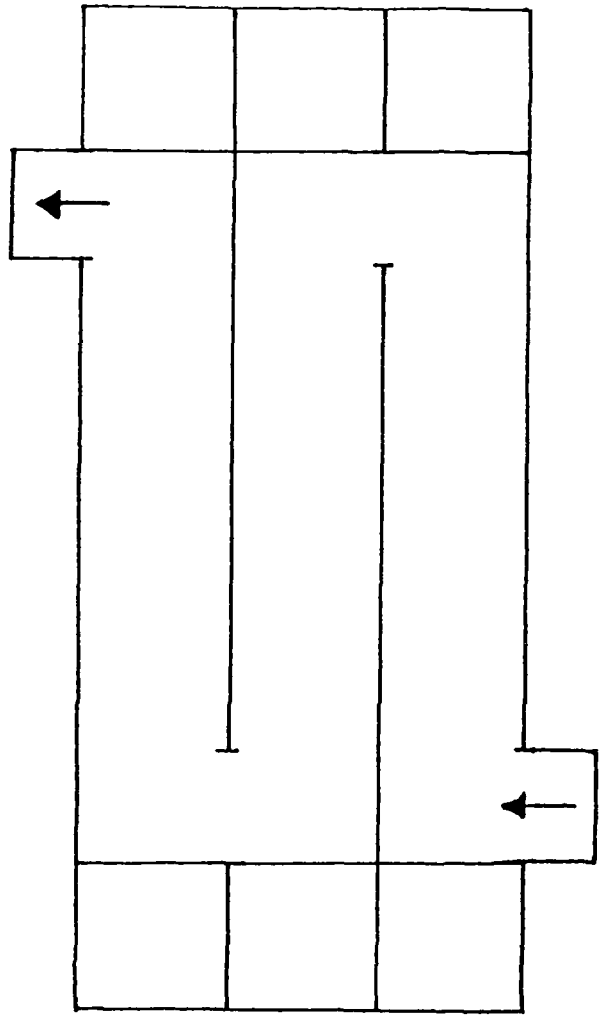
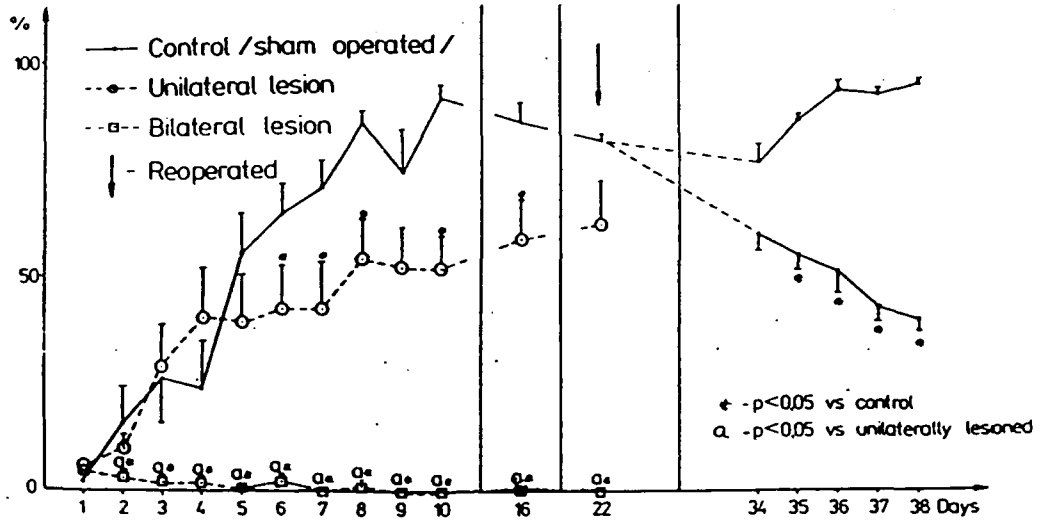
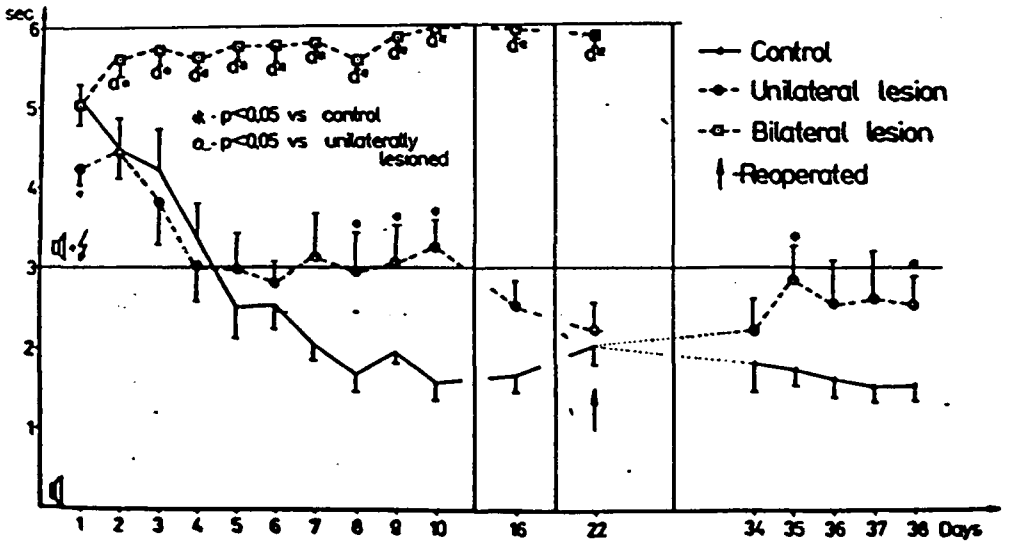


FIG. 9

NUMBER OF CORRECT REACTION / AFTER CONDITIONAL ONLY /



TIME OF REACTION / LATENCY PERIOD / OF ANIMALS ON THE CONDITIONAL AND REINFORCEMENT STIMULUS



L. Freysz | J.N. Hawthorne | G. Toffano

NEUROCHEMICAL ASPECTS OF PHOSPHOLIPID METABOLISM

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MODULATION OF PHOSPHOLIPID METABOLISM IN RAT STRIATUM BY THE CORTICOSTRIATAL PATHWAY

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Surgical undercutting of cortical afferents and subsequent interruption of excitatory input increased PEMT activity by 30-40% in rat striatal synaptosomes as early as three days after lesion, and lasted 60 days. The increase was protein-dependent and lesion-specific. The specific antagonist of NMDA receptors, APV, increased PL methylation in non-lesioned striata, mimicking the effect of decortication, while NMDA counteracted the decortication induced increase in PEMT activity. In sham-lesioned rats DA stimulated PL methylation, but it did not further stimulate PEMT activity in decorticated rats. In addition, DA antagonists reversed the increase in PL methylation in lesioned rats at concentrations that did not block DA-stimulated PL methylation in sham-lesioned rats. These data suggest a modulatory role of the cortex on PEMT activity in rat striatum.

In recent years, the methylation of phosphatidylethanolamine (PE) to phosphatidylcholine (PC) has been shown to play an important role in the transduction of receptor-mediated signals through membranes of a variety of cells (see references in Crews, 1985). It has also been found that synaptosomes from rat brain are enriched in phospholipid methyltransferases, EC 2.1.1.17, (PEMT) and that DA has an effect on these enzymes in the striatum (Leprohon et al., 1983) an area which stands out in the brain as receiving a massive excitatory input originating in the frontal cortex. However, so far no information is available on the influence on PEMT activity of the striatal excitatory aminoacids associated with the frontal cortex input. We thus set out to determine whether interruption of the excitatory pathway, hence loss of the excitatory neurotransmitter, presumably glutamate, affects PL methylating reactions and whether it influences DA's effect on PEMT.

Female CD-COBS rats, b.w. 175 g. (Charles River Italy, Calco, Italy) were used. Bilateral frontal decortication (DC) was made in lightly etherized rats by undercutting the cortex as previously reported (Consolo et al., 1986). The uptake of (^{3}H)glutamate in crude homogenate preparations (P_2 fraction) of randomly selected DC rats was measured in order to assess the specificity of the lesion. Effective lesions were produced in more than 90% of the rats in which (^{3}H)glutamate uptake was reduced by 60%.

APV = 2-Amino-5-phosphonovaleric acid; DC = decortication; DA = dopamine; PC = phosphatidylcholine; PE = phosphatidylethanolamine; PEMT = phosphatidylethanolamine N-methyltransferase; PMME = phosphatidyl N-monomethylethanolamine; PDME = phosphatidyl N,N-dimethylethanolamine; NMDA = N-methyl-D-aspartic acid; SAM = S-adenosylmethionine.

Neurochemical Aspects of Phospholipid Metabolism. L. Freysz, J.N. Hawthorne, G. Toffano (eds) Fidia Research Series, vol. 20, Liviana Press, Padova © 1989

Animals were killed by decapitation 3, 14 and 60 days after lesion and striata were quickly removed. Striatal synaptosomes were prepared by sucrose gradient centrifugation according to Dodd et al. (1981). PEMT activity was measured by incubating different samples of synaptosomes for 30 min at 37°C in the presence of 1.2 to 100 μM (methyl-^{3}H) S-adenosylmethionine (SAM, 2.5 μCi/assay) (Tacconi and Wurtman, 1985). After lipid extraction, methylated PLs (PC, PDME, and PMME) were separated by TLC and their radioactivity measured. Protein was measured according to Lowry et al. (1951). The data were analyzed by one-way ANOVA and Tukey's test for unconfounded means.

Striatal synaptosomes of sham-lesioned rats and 3, 14 and 60 day DC were incubated with 55 μM (methyl-^{3}H) SAM (Table 1). Decortication resulted in fairly uniformly increased (methyl-^{3}H) incorporation into all three methylated PLs (PMME, PDME and PC) as early as three days after lesion, but at later times the increase was mainly in PMME and PDME; PC, the last methylated product, was not influenced. Total methyl-^{3}H incorporation was 16.1 ± 0.3 pmol/mg protein/min in DC rats versus 12.2 ± 0.8 in sham ones at three days and was still up 25% 14 and 60% days after lesion.

TABLE 1

Effect of frontal decortication on phospholipid methylation in rat striatal synaptosomes

Days after lesion	Methyl-^{3}H incorporation into phospholipids (pmol/mg protein/30 min)			
	PMME	PDME	PC	Total
Sham	6.2 ± 0.3	4.8 ± 0.3	1.2 ± 0.2	12.2 ± 0.8
3	8.3 ± 0.1*	5.7 ± 0.1*	2.1 ± 0.1*	16.1 ± 0.3*
14	10.2 ± 0.1*	6.8 ± 0.3*	1.3 ± 0.2	18.3 ± 0.7*
60	7.7 ± 0.1**	6.8 ± 0.3*	1.2 ± 0.1	15.7 ± 0.5**

The data are means and S.E. (n = 5); *$p < 0.01$ versus respective control; **$p<0.05$ determined by one-way ANOVA and Tukey's test for unconfounded means

Methylated PL production was protein-related, in a range of protein concentrations from 35 to 140 μg/assay, both in sham-operated and DC rats; however, DC synaptosomes produced 30-40% more methylated PLs than the sham ones at all protein concentrations (data not shown).

Methylated PL production in DC-synaptosomal preparations was dependent on SAM concentration, as in sham-operated ones. However, in the sham-operated membrane preparations, formation of (^{3}H) PMME , the first methylated product, reached saturation around 30 μM SAM while in DC ones it did not, even at 100 μM SAM. The positive effect of DC on PMME production was more evident at a higher SAM concentration. PDME and PC production followed the same pattern, although PC was affected less.

The changes in PEMT activity after PC seemed to be specific for this lesion, since chemical degeneration of the medial and dorsal raphe serotoninergic pathways to the striatum, which reduced striatal 5-HT more than 90%, did not affect PL methylation; total methyl-^{3}H incorporation being 4.6 ± 0.3 and 4.7 ± 0.2 pmol/mg/protein/30 min for sham operated and lesioned animals respectively.

The results all together suggest that the excitatory aminoacid input in the striatum might, under physiological conditions, have an inhibitory effect on PL

methylation. This hypothesis is further supported by the following findings: when excitatory aminoacid receptors were blocked by 2-amino-5-phosphonovaleric acid (APV) (1 mM and 500 μM), a selective antagonist of N-methyl-D-aspartate type receptors (NMDA), PEMT activity increased 30% (Fig. 1) in sham-operated rats, an effect similar to that produced by interruption of the cortical glutamatergic pathway. On the other hand, addition of the NMDA receptor agonist, NMDA(0.5 and 1 mM) although ineffective in sham-operated rats, significantly reduced the PL methylation rise in DC rats.

It is conceivable that the neurons intrinsic to the striatum may possibly by maintained in a functional state by a balance between the excitatory input from the cortex and the dopaminergic influence from the substantia nigra. Loss of the glutamatergic influence could shift this balance, so all the activities under the regulation of the dopaminergic input (including methylation) are affected. Thus we found that DA (10 and 50 μM) stimulated PL methylation in sham-operated rats, but not in DC-rats. Dopamine antagonists such as haloperidol and SCH 23390 reversed the increase in PL methylation in lesioned rats at concentrations (100 and 10 μM, respectively), that did not block DA-stimulated PL methylation in sham-lesioned rats. These results confirm that the over stimulation is due to increased dopaminergic tone.

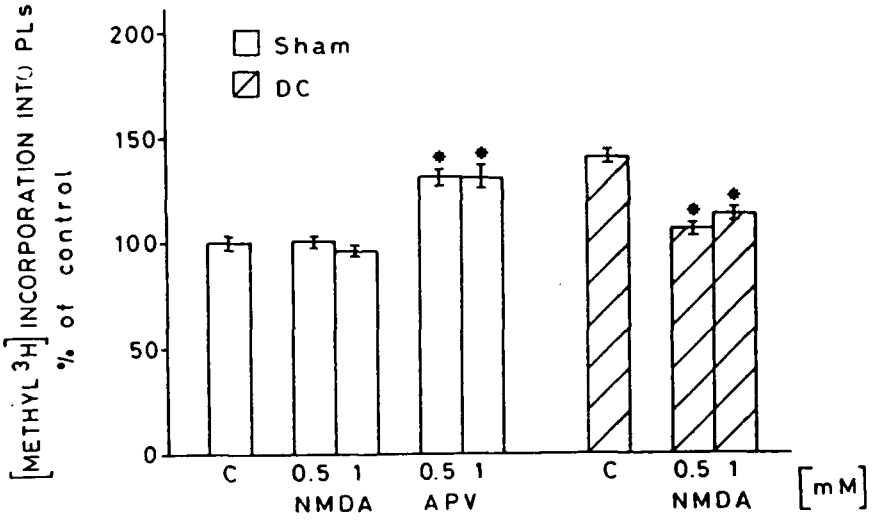

Figure 1

Effect of NMDA and APV (0.5 and 1 mM) on PEMT activity in sham-operated ☐ and 14-day DC ▨ striata. Data are percentage of total methyl-^{3}H incorporated into PLs, in comparison to sham controls (n = 5).
*p <0.01 versus respective control, as determined by one-way ANOVA and Tukey's test for unconfounded means.

Little is known about the physiological role of PL methylation in rat brain. It is conceivable that choline produced by the PEMT pathway sustains the striatal cholinergic activity depressed by DC, as reflected by the reduced high-affinity uptake of choline and the lower acetylcholine turnover (Consolo et al., 1986). This hypothesis is in agreement with the suggestion by Blusztajn et al. (1986) that PL methylation may provide newly formed choline for cholinergic neurons and may therefore regulate acetylcholine biosynthesis.

In conclusion, this study gives the first evidence that the corticostriatal pathway inhibits PL methylation in striatal synaptosomes. The findings suggest that under physiological conditions the frontal cortex influences PL methylation through activation of NMDA receptors within the striatum.

Acknowledgement

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Treatment with Oxiracetam or Choline Restores Cholinergic Biochemical and Pharmacological Activities in Striata of Decorticated Rats

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Abstract: Interruption of the corticostriatal pathway by undercutting the frontal cortex resulted after 2 weeks in a 40% reduction of basal acetylcholine (ACh) release in vivo, and in inhibition of the striatal sodium-dependent high-affinity uptake of choline (SDHACU) to the same extent. The lesion, too, completely prevented the rise (about 35%) in striatal ACh content induced by oxotremorine and apomorphine acting at muscarine and dopamine receptors, respectively. Acute intraperitoneal injections of 100 mg/kg of either oxiracetam or choline chloride resulted in time-dependent recovery of ACh output from the striata of decorticated rats to control levels. Oxiracetam also normalized the ex vivo striatal SDHACU activity of decorticated rats 2 h after administration without any effect in sham-operated rats. Oxiracetam or choline chloride administered before oxotremorine (0.8 mg/kg, i.p.) or apomorphine (1 mg/kg, i.p.) reinstated the ACh-increasing effect of these agonists. It is suggested that choline chloride acts directly simply by being the precursor for ACh, whereas oxiracetam may act indirectly, possibly by increasing the availability of choline chloride for ACh synthesis. Furthermore, the frontally decorticated rat could constitute a useful model for studying means to restore the deficit in striatal cholinergic neurotransmission. **Key Words:** Striatum—Oxiracetam—Choline—Decorticated rats—Acetylcholine release in vivo—Acetylcholine content—Choline uptake. **Consolo S. et al.** Treatment with oxiracetam or choline restores cholinergic biochemical and pharmacological activities in striata of decorticated rats. *J. Neurochem.* **54**, XXX–XXX (1990).

The striatum, unlike many other parts of the brain, receives a massive excitatory input originating in the frontal cortex as indicated by electron microscopic (Hassler et al., 1978), electrophysiological (Spencer, 1976), and neurochemical studies (Divac et al., 1977; McGeer et al., 1977; Reubi and Cuenod, 1979). The cholinergic neurons intrinsic to the striatum appear to be particularly influenced by this pathway. Removal of excitatory corticostriatal afferents, by cortical ablation or undercut, results in a reduction in the rate of acetylcholine (ACh) synthesis (Wood et al., 1979) and in an inhibition of sodium-dependent high-affinity choline uptake (SDHACU) (Simon, 1982), indicating that the lesion depresses basic cholinergic activity in the striatum. As regards striatal cholinergic neuropharmacology, cortical deafferentation completely prevents the large rise in striatal ACh content induced by a number of agonists capable of depressing cholinergic activity through receptor-mediated responses (Consolo et al., 1986). On the other hand, the activity of choline acetyltransferase, a specific marker for cholinergic neurons, is not affected by the lesion (Ladinsky et al., 1987), suggesting that the striatal cholinergic system remains intact after long-term decortication. Therefore, even when its activity is depressed, this group of neurons remains potentially functional and capable of responding to certain stimuli.

We have some evidence that the neuropharmacological activity of apomorphine (APO) can be restored in decorticated (DC) rats by choline chloride (Ch)

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Abbreviations used: ACh, acetylcholine; ANOVA, analysis of variance; APO, apomorphine; Ch, choline chloride; DC, decorticated; OTMN, oxotremorine; OXI, oxiracetam; SDHACU, sodium-dependent high-affinity choline uptake.

B5808-104, 105 D 10685 DC

(Consolo et al., 1986), a precursor of ACh that has attracted widespread interest in the treatment of neurological diseases for its property of enhancing ACh synthesis (Corkin et al., 1982; Domino et al., 1982; Wurtman, 1985), and by oxiracetam (OXI) (Salmoiraghi et al., 1987), a second-generation nootropic drug in the 2-pyrrolidinone chemical class whose therapeutic value is currently being investigated (Itil et al., 1982; Moglia et al., 1984).

In order to establish whether and to what extent these drugs restore cholinergic function in DC rats, we investigated whether Ch and OXI could promote the recovery of the basic biochemical cholinergic processes and the cholinergic neuropharmacological activity of APO and oxotremorine (OTMN) in striata of DC rats.

MATERIALS AND METHODS

All reagents used were analytical grade. The following drugs were obtained from commercial sources: OTMN sesquifumarate (Sigma, St. Louis, MO, U.S.A.); *R*-APO (Sandoz, Basel, Switzerland); and Ch (Sigma). OXI was generously supplied by I.S.F. (Trezzano sul Naviglio, Milan, Italy). All the drugs were dissolved in distilled water and administered as specified in the figure legends. Doses always refer to the salt form.

Animals

Female CD/COBS rats (180–220 g body weight; Charles River, Calco, Italy) were used. The animals were given free access to water and food and were housed in groups of four in Makrolon cages under standard conditions of humidity (60%), room temperature (22°C), and 12-h light/12-h dark cycles for at least 4 days before the experiment.

Frontal cortex lesion

Pentobarbital-anesthetized rats (35 mg/kg) were frontally DC by undercutting the cortex. The animals were positioned in a stereotaxic apparatus, and the skull was opened by a 6-mm lateral cut along the frontal–temporal suture starting 2 mm below the bregma. A horizontal undercut of the right hemisphere or both hemispheres, depending on the experiment, was then made with a glass knife fashioned from a cover slip. In sham-operated animals, the skull was opened, but no lesion was made. Bilaterally DC animals were used for the determination of ACh release in vivo and SDHACU activity ex vivo, where unilaterally DC rats were used for determination of tissue ACh content. The experiments were performed 14 days after the lesion. The specificity of the lesion was assessed in randomly selected groups of four rats per experiment by measuring the uptake of [^{3}H]glutamate in crude homogenate preparations according to the method of Divac et al. (1977). Effective lesions were produced in more than 90% of the rats in which the [^{3}H]glutamate uptake was reduced by 60% 14 days after lesion.

Dialysis probe implantation and perfusion

The rats were anesthetized with chloral hydrate (400 mg/kg, i.p.) and placed in a stereotaxic frame. Dialysis tubing (220 μm internal diameter and 310 μm external diameter; AN 69 membrane, Dasco, Bologna, Italy), with a molecular weight cutoff of >15,000, was inserted transversally through both striata. The dialysis tubing was covered with Super-Epoxy glue along its whole length, except for the parts which corresponded to the areas of interest (7.5 mm). The procedure used to insert the dialysis probe was essentially the same as previously described by others (Imperato and Di Chiara, 1984; Zetterstrom and Ungerstedt, 1984). The coordinates for implanting the probes were A 1.5 mm from bregma and V 5.3 mm from temporal bone (Konig and Klippel, 1963).

Recovery through the dialysis tubing, prepared in the same manner as that implanted through striata, was determined in vitro. Dialysis tubing was placed in Eppendorf vials filled with Ringer's solution containing 100 MBq of [^{3}H]ACh in a final concentration of 70 μ*M*, equivalent to the ACh content measured in striatum of microwaved rats, and perfused with Ringer's solution (2 μl/min) at room temperature. The average [^{3}H]ACh recovery, determined in 10-min samples collected over a 120-min perfusion period, was calculated as a percentage of the radioactivity in the solution outside the tubing.

On the day after implantation, each rat was placed in a Plexiglas cage (25 × 25 × 30 cm). The dialysis probe was connected to a microinfusion pump (Sage Instruments, Model 355, Orion Research Inc., Cambridge, MA, U.S.A.) by polyethylene tubing and perfused at a constant rate of 2 μl/min with Ringer's solution (147 m*M* NaCl, 3.4 m*M* $CaCl_2$, and 4.0 m*M* KCl), containing 10 μ*M* physostigmine sulfate. The pH was adjusted to 7 with NaOH. The perfusate was discarded during the first 30 min and then collected at 10-min intervals in small ice-cooled polyethylene tubes containing 5 μl of 0.5 m*M* HCl to prevent ACh hydrolysis. At the end of the collection, the perfusate samples were immediately frozen on dry ice and lyophilized.

On completion of the dialysis experiments, the brain was rapidly dissected out and, after a coronal cut, frozen and sliced by a cryostat to check the location of the dialysis tubing. Experiments in which the dialysis tubing was not placed correctly were discarded.

Assays of ACh

ACh content in perfusates. ACh content in the perfusate was quantified by a sensitive and specific radioenzymatic method previously described (Consolo et al., 1987; Wu et al., 1988). Briefly, the method consists of the conversion of Ch to phosphorylcholine in the presence of choline phosphokinase and ATP, the enzymatic hydrolysis of ACh to Ch and acetic acid, and the reacetylation of the resulting Ch to [^{3}H]ACh with the addition of [^{3}H]acetyl-CoA (74–92.5 GBq/mmol) and acetyl-CoA:choline *O*-acetyltransferase (EC 2.3.1.6.). The resulting [^{3}H]ACh was separated from [^{3}H]acetyl-CoA by extraction into tetraphenylboron-containing ketone phase by liquid–liquid ion-exchange chromatography and counted for radioactivity (Fonnum, 1975). Phosphorylation, hydrolysis, and acetylation reactions were validated routinely. The concentration of ACh in each sample was calculated by linear regression based on the radioactivity of the standards (linear from 1 to 25 pmol of ACh with the slope equal to 5,800 net dpm/pmol at a [^{3}H]acetyl-CoA specific activity of 74 GBq/mmol). The coefficient of variation of the replicate perfusate samples or ACh standard was about 3%. None of the drugs used in this study caused interference with the assay.

ACh content in tissues. The rats were killed by fast-focussed microwave irradiation to the head (1.3 kW, 2.45 GHz, 4 s). The brain was quickly removed, the striatum was dissected, and the ACh content was measured. The tissues were homogenized in 1 *M* formic acid/acetone (15:85) before mea-

surement of ACh by the radioenzymatic method of Saelens et al. (1970) with modifications (Ladinsky et al., 1976).

SDHACU activity

Striatal homogenates were centrifuged at 1,000 *g* for 10 min at 4°C. The pellet (P_1) was discarded. The resulting supernatants were centrifuged at 17,000 *g* for 15 min to obtain crude mitochondrial pellets (P_2). SDHACU activity was measured in the P_2 fraction according to the method of Atweh et al. (1975), except that the reaction was terminated by filtration through 0.65-μm Sartorius cellulose nitrate filters under negative pressure. After washing three times with 1 ml of cold Na^+-free Krebs or normal Krebs solution, as appropriate, the filters were added to 10 ml of Filter Count TM (Packard) for scintillation counting. [^{3}H]Ch (80 Ci/mmol) was added at the final concentration of 0.03 μM.

Statistics

Different types of analysis of variance [two-way ANOVA (4 × 2) and Splitplot test] and multiple comparison tests (Tukey's test for unconfounded means and Dunnett's test) were used, as specified in the figure legends.

RESULTS

Restoration of ACh release in vivo by OXI or Ch in DC animals

The extracellular ACh content in vivo was measured in striata of 14-day DC and sham-operated rats, using the microdialysis technique. The results are shown in Fig. 1. The ACh levels in the 10-min perfusate samples are given as values uncorrected for the recovery, which was 49.2% for a probe 7.5 mm long.

The ACh output was constant over at least 240 min in the sham-operated and DC rats. The average ACh content in the perfusate of sham-operated rats was 8.1 ± 0.2 pmol/10 min, and this was significantly lowered, to 5.3 ± 0.9 pmol/10 min ($p < 0.01$), in DC rats given saline (Fig. 1).

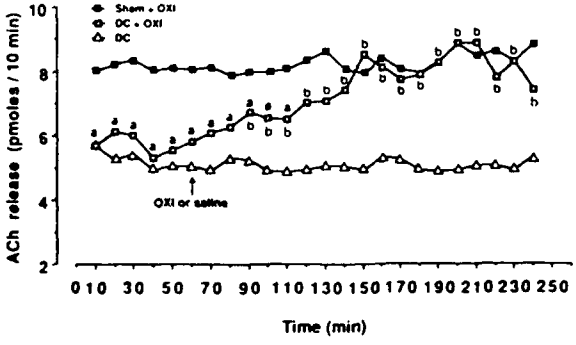

FIG. 1. OXI administration restores normal striatal ACh release in vivo in DC rats. The experiments were done 14 days after sham operation or bilateral frontal decortication and 24 h after dialysis tube implantation. Perfusate was collected for 60 min (6 × 10-min fractions) before injection of OXI (100 mg/kg, i.p.) or saline (shown by the arrow). The data points (means of six rats) represent the ACh content in each 10-min fraction and are expressed as pmol/10 min. Variability between values did not exceed 5%. ACh release in the sham + OXI group was not significantly different from that of sham + saline at any time (curve not shown). ACh release in the DC group was significantly ($p < 0.01$) different from that of the sham + OXI group at all times, as determined by Dunnett's test. Interactions: DC + OXI versus sham + OXI, $^a p < 0.01$; DC + OXI versus DC + saline, $^b p < 0.01$; Splitplot test and Tukey's test for unconfounded means.

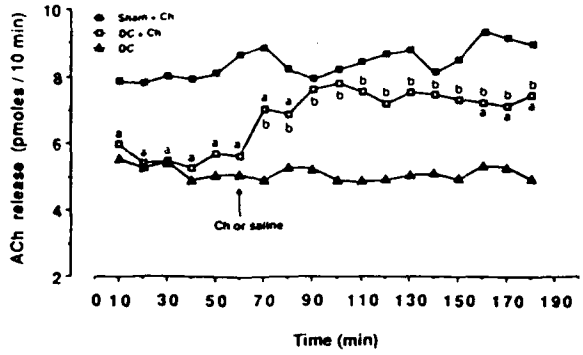

FIG. 2. Ch administration restores striatal ACh release in vivo in DC rats. The experiments were done 14 days after sham operation or bilateral frontal decortication and 24 h after dialysis tube implantation. Perfusate was collected for 60 min (6 × 10-min fractions) before injection of Ch (100 mg/kg, i.p.) or saline (shown by arrow). The data points (means of six rats) represent the ACh content in each 10-min fraction and are expressed as pmol/10 min. Variability between values did not exceed 5%. ACh release in the sham + Ch group was not significantly different from that of sham + saline at any time (curve not shown). ACh release in the DC group was significantly ($p < 0.01$) different from that in the sham + Ch group at all times, as determined by Dunnett's test. Interactions: DC + Ch versus sham + Ch, $^a p < 0.01$; DC + Ch versus DC + saline, $^b p < 0.01$; Splitplot test and Tukey's test for unconfounded means.

An intraperitoneal dose of OXI (100 mg/kg) did not significantly alter the ACh output from striata of sham-operated rats at any time over the entire collection period; the average ACh content for this group (sham + OXI) was not significantly different (8.4 ± 0.6 pmol/10 min) from the sham-operated group of rats given saline, so only the ACh release curve of sham + OXI is presented in Fig. 1.

In DC rats, the nootropic drug induced a time-dependent recovery of ACh output from the striata. Starting 30 min after drug injection, ACh release was already significantly ($p < 0.01$) higher in this group of lesioned rats than in the group of lesioned animals receiving saline, and the rate of ACh release continued to rise gradually, with complete recovery about 80 min post OXI injection. The full effect lasted at least 100 min longer (Fig. 1).

Pretreatment with choline (100 mg/kg, i.p.), the ACh precursor, did not influence the ACh output from striata of sham-operated rats, but completely overcame the reduction in extracellular ACh content observed in DC rats (Fig. 2). A substantial recovery effect started 10 min after Ch administration when the ACh content of treated lesioned rats was 42% higher than that in untreated DC lesioned ones at the corresponding time. ACh release appeared to plateau about 30 min after the start of treatment, when control values were reached. The effect of Ch lasted at least 80 min more.

Restoration of SDHACU activity by OXI in DC animals

The SDHACU by the P_2 fraction of striatal homogenates was reduced by about 35% 14 days after frontal decortication compared to the sham-operated animals (from 0.8 ± 0.06 to 0.5 ± 0.03 nmol of Ch taken up/min/g of protein; Table 1). OXI normalized the striatal SDHACU activity of DC rats 120 min after a dose of 100 mg/kg, but had no effect on SDHACU activity of sham-operated rats.

Restoration of the ACh-increasing effect of OTMN or APO in DC rats by OXI or Ch

OTMN, a typical muscarinic agonist, at the dose of 0.5 mg/kg, i.p., after 20 min, and APO, a D_1-D_2 dopaminergic agonist, at the dose of 1.0 mg/kg, i.p., after 30 min, increased striatal ACh content in sham-operated rats by about 30%. Decortication by itself did not affect striatal ACh content, but it completely prevented the increase in cholinergic effect of both OTMN and APO (Fig. 3).

OXI (100 mg/kg, i.p., after 120 min) did not by itself affect striatal ACh content in either sham-operated or DC rats, but when it was administered prior to OTMN and APO (100 and 90 min, respectively), it restored the ACh-increasing effect of these drugs.

In a dose-response study, it was found that at the higher dose of 300 mg/kg, OXI had a similar restorative effect on the OTMN- and APO-induced ACh increase, but not at the lower doses of 30 and 60 mg/kg (data not shown). By itself, OXI affected neither striatal ACh nor striatal Ch content in either sham-operated or DC rats 120 min after any of the doses tested (data not shown).

Ch (100 mg/kg) given 10 min before OTMN or APO reinstated the ACh-increasing effect of the two agonists in DC rats (Fig. 4). At the dose of 100 mg/kg 10 min before OTMN or APO, Ch did not significantly affect striatal ACh in sham-operated or DC animals (data not shown), but raised Ch about 60% in both groups (Table 2). The effect was transient, because at 30 and 80 min it was no longer detectable.

TABLE 1 *OXI restores SDHACU activity in striata of bilaterally DC rats*

	SDHACU (nmol of Ch/min/g of protein)	
	Sham	DC
Vehicle	0.8 ± 0.06	0.5 ± 0.03[a]
OXI	0.7 ± 0.06	0.9 ± 0.06

The experiments were done 14 days after bilateral frontal decortication. OXI (100 mg/kg) was administered intraperitoneally 120 min before Ch uptake was measured, as described in Materials and Methods. The data are means ± SEM of eight animals.

[a] $p < 0.01$ versus sham group [$F(1,25) = 22.8$; ANOVA (2 × 2) and Tukey's test for unconfounded means]

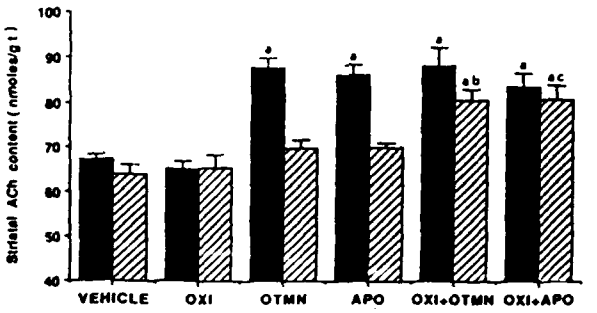

FIG. 3. Blockade of the ACh-increasing effect of OTMN and APO in striata of DC rats and restoration by OXI. The experiments were done 14 days after unilateral (right side) frontal decortication. The animals were killed by microwave irradiation to the head 120 min after OXI (100 mg/kg, i.p.), 20 min after OTMN (0.5 mg/kg, i.p.), and 30 min after APO (1 mg/kg, i.p.), and the right striata were removed for determination of ACh content. The data are means ± SEM (n = 6–12 rats). The solid columns show the results for sham-operated rats and hatched columns those for DC ones. Data were analyzed statistically by two-way ANOVA, followed by Tukey's test for unconfounded means; $^a p < 0.01$ versus respective vehicle-treated group. Interactions: OXI + OTMN versus OTMN, $F(1,40) = 11.7$, $^b p < 0.01$; OXI + APO versus APO, $F(1,24) = 17.9$, $^c p < 0.01$.

DISCUSSION

The results of this study provide clear evidence that the striatal cholinergic interneurons, markedly impaired in their biochemical activities and pharmacological sensitivity with the loss of the excitatory corticostriatal input, can be restored to normal activity by appropriate pharmacological maneuvers. Acute treatments with Ch and OXI normalized the reduction in ACh release in vivo and in SDHACU activity ex vivo.

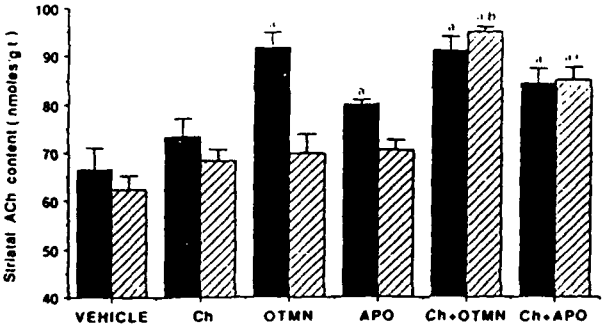

FIG. 4. Blockade of the ACh-increasing effect of OTMN and APO in DC rats and restoration by Ch. The experiments were done 14 days after unilateral frontal decortication. Ch (100 mg/kg, i.p.) was administered 10 min before either OTMN (0.5 mg/kg, i.p.) or APO (1 mg/kg, i.p.). The animals were killed by microwave irradiation to the head 20 min after OTMN and 30 min after APO. The solid columns show the results for sham-operated rats and hatched columns those for DC ones. The data are means ± SEM (n = 6–8 rats). Data were analyzed statistically by two-way ANOVA, followed by Tukey's test for unconfounded means; $^a p < 0.01$ versus respective vehicle-treated group. Interactions: Ch + OTMN versus OTMN, $F(1,24) = 15.1$, $^b p < 0.01$; Ch + APO versus APO, $F(1,16) = 14.9$, $^c p < 0.01$.

TABLE 2. *Time course of the effect of Ch on striatal Ch content in sham-operated and in DC rats*

Time (min)	ACh (nmol/g/min)	
	Sham	DC
0	25.2 ± 1.4	23.7 ± 3.0
10	41.2 ± 4.0[a]	35.7 ± 4.0[a]
30	29.5 ± 1.8	26.6 ± 3.4
80	27.5 ± 1.8	28.2 ± 1.3

The data are means ± SEM of six animals. Ch was administered intraperitoneally at a dose of 100 mg/kg.
[a] $p < 0.01$ versus respective control group (Dunnett's test).

and reinstated cholinergic sensitivity to APO and OTMN in striata of DC rats. Because the two compounds restored basic cholinergic processes closely connected with ACh synthesis, they must also have accelerated the depressed turnover of ACh in striata of DC rats, although this parameter was not measured directly.

There are indications that an increased supply of Ch in the brain can increase the amount of ACh synthesized and released from cholinergic terminals (Maire and Wurtman, 1985). Indeed, it has been proposed (Ansell and Spanner, 1979) that Ch is present in the brain at a concentration well below that needed to saturate choline acetyltransferase, the ACh-synthesizing enzyme, and thus the net rate of synthesis will depend primarily on availability of the precursor. In addition, Ch is known to have direct, though weak, muscarinic receptor agonist activity, as shown in neuropharmacological (Consolo et al., 1979; Ladinsky et al., 1979), binding (Speth and Yamamura, 1979), and electrophysiological (Krnjevic and Reinhardt, 1979) studies, which may complement its indirect action.

On the other hand, although the mechanism of action of OXI is not yet well understood, there is experimental evidence that it could act by increasing SDH-ACU, a process believed to represent the rate-limiting step in the synthesis of ACh (Atweh and Kuhar, 1976; Antonelli et al., 1981). Thus, OXI promotes the recovery of cholinergic activity in the CNS altered by treatment with the antimuscarinic agent, scopolamine (Spignoli and Pepeu, 1987), and in the periphery it antagonizes the lethal effects of the neuromuscular blocking agent and choline-uptake inhibitor, hemicholinium (Hall and Von Voigtlander, 1987).

These observations indicate that OXI and Ch share the potential for enhancing cholinergic neurotransmission by promoting the availability of ACh precursor in the brain. In this regard, it was shown that both OXI and Ch cross the blood–brain barrier. OXI distributes as unchanged drug in the brain over 30 min (Ponzio et al., 1988), whereas Ch reaches the brain sooner (Haubrich et al., 1979; Pardridge et al., 1979). This could account for the later onset of the recovery effect of OXI on ACh release than that of Ch.

A distinguishing feature of OXI is that it displays its action only in animals with impaired cholinergic function. Thus, OXI, even up to the dose of 300 mg/kg, does not have any effect on the striatal cholinergic system of sham-operated rats, whereas Ch at the dose of 100 mg/kg used in this study increases striatal Ch content transiently both in sham-operated and DC rats, and at higher doses it can raise the ACh content (Cohen and Wurtman, 1975; Haubrich et al., 1975*a*; Consolo et al., 1986), ACh turnover (Haubrich et al., 1975*b*; Eckernas et al., 1977), and ACh release in vitro (Maire and Wurtman, 1985) of naive animals. This is in agreement with findings of others that the nootropic drug is more effective in animal models in which learning and memory are impaired either acutely (electroshock, hypoxia) or chronically (cerebrovascular lesions, microencephalic or aged rats) (Banfi and Dorigotti, 1986) and with biochemical experiments showing that OXI prevents electroshock- or scopolamine-induced decrease in brain ACh, but has no effect in control animals (Spignoli and Pepeu, 1986, 1987).

The neurons intrinsic to the striatum may possibly be kept in a functional state by a balance between the excitatory input from the cortex and inhibitory dopaminergic and serotonergic afferents from the substantia nigra and dorsal raphe (Ladinsky et al., 1987). Loss of excitatory influence by decortication shifts the balance in favor of the inhibitory tone, and drugs that normally slow down cholinergic activity are no longer active, e.g., OTMN, acting at muscarinic receptors, or APO, acting at dopaminergic receptors. Therefore, pretreatment with OXI or Ch, by restoring the normal basic biochemical processes, may have raised the activity state of the neurons, so the ACh-increasing effect of both agonists is reinstated. Restoration of either OTMN or APO cholinergic activity by OXI and Ch suggests that the muscarinic receptors are not involved in the mechanisms by which these compounds stimulate recovery.

The data taken together indicate that Ch most likely acts directly simply by being the precursor for ACh, whereas OXI appears to act indirectly, possibly by increasing the availability of Ch for ACh synthesis. In accordance with this hypothesis is the finding (Trovarelli et al., 1986) that OXI improves the in vitro and in vivo synthesis of phospholipids impaired by aging, and in particular, the phosphatidylcholine pool proposed as a "reservoir" to generate choline for the synthesis of ACh (Blusztajn et al., 1986; Lakher et al., 1986). Interestingly, it was reported that ACh can be synthesized from Ch derived from the breakdown of endogenous phosphatidylcholine formed de novo by the stepwise methylation of phosphatidylethanolamine (Lakher et al., 1986). In accordance with this is the finding that, in striatum of DC rats, the transmethylation pathway is enhanced (Tacconi et al., 1988), possibly to sustain the striatal cholinergic activity depressed by the lesion. However, whether the effect of OXI occurs in the transmethylation pathway or in others

known to exist in the brain (Blusztajn et al., 1986; Lakher et al., 1986), e.g., CDP-choline and the base exchange pathways, needs to be clarified.

In summary, our findings indicate that the loss of ACh release in vivo and the SDHACU activity in DC rats can be reversed with OXI and Ch. This repletion is associated most likely with enhanced ACh formation. As a consequence, the cholinergic neuropharmacological effects of APO and OTMN are also reinstated in DC rats.

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REDUCTION OF [3H]HEMICHOLINIUM BINDING AND IN VIVO ACETYLCHOLINE RELEASE IN RAT STRIATUM BY CORTICAL DEAFFERENTATION: RESTORATION STUDIES WITH CHOLINE AND OXIRACETAM.

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Frontal deafferentation of the rat striatum reduces tha tone of striatal cholinergic neurons. This effect has been determined by the measurement of several cholinergic parameters including in vivo acetylcholine (ACh) release by the microdialysis technique and [^{3}H]hemicholinium-3 ([^{3}H]HCh-3) binding to sodium dependent high affinity choline uptake (SDHACU) sites by biochemical and autoradiographic techniques. The lesion resulted after twoo weeks in a 30-40 % reduction of both parameters. Acute i.p. injections of 100 mg/kg of oxiracetam (OXI) induced time-dependent recovery of ACh output from the striata of decorticated rats . Starting 30 min after drug injection, ACh release was already significantly higher in the OXI-treated group compared to the saline-treated group and the rate of ACh release continued to rise gradually, with complete recovery within 80 min after drug injection. The full effect lasted at least 100 min longer. Similar effect with different time-course was observed with choline chloride treatment (100 mg/kg, i.p.). OXI also normalized the [3H]HCh-3 striatal binding of decorticated rats 2 h after its administration without any effect in sham-operated rats. This result is consistent with the ability of OXI to restore the SDHACU activity in cortical deafferented striatum. It is possible that the increase of Ch aviability for the ACh synthesis is the common mechanism used by these drugs to restore the cholinergic function. The results suggest, too, that the striatum of decorticated rats could constitute a useful model for studyng means to restore the deficit in cholinergic neurotransmission.

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